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Award Number: DAMD17-01-1-0343

TITLE: The Roles of FGF-2, TGF Beta and TGF Beta Receptor 2 In
Breast Cancer Dormancy

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REPORT DATE: July 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20040105 053

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2003	3. REPORT TYPE AND DATES COVERED Final(1 Jul 2001 - 30 Jun 2003)	
4. TITLE AND SUBTITLE The Roles of FGF-2, TGF Beta and TGF Beta Receptor 2 In Breast Cancer Dormancy			5. FUNDING NUMBERS DAMD17-01-1-0343	
6. AUTHOR(S) Robert Wieder, M.D., Ph.D				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Medicine and Dentistry of New Jersey Newark, NJ 07103-2714 E-Mail: wiederro@umdnj.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Basic Fibroblast growth factor is associated with ductal morphogenesis in mammary duct development and its expression is lost in breast cancer. We determined the expression of FGF-2 during different stages of mammary carcinogenesis in archived surgical specimens. FGF-2 was expressed in the majority of specimens from normal, benign and atypical hyperplasia, fibrocystic disease and carcinoma in situ, but its expression frequency dropped significantly in invasive cancer. Enforced re-expression of FGF-2 in breast cancer cell lines suggested a causal role for a more differentiated phenotype, including decreased motility and invasiveness. The decreased motility was associated with constitutive and omnidirectional focal adhesion complex activation and rearrangement of actin filaments. In a separate project, we investigated a potential mechanism for dormancy and survival of microscopic metastases in the bone marrow. Our model suggests that FGF-2 in the marrow inhibits proliferation of well-differentiated breast cancer cells and inhibits their survival. FGF-2 induces the overexpression of integrins alpha 5 and beta 1, which in turn bind fibronectin in the microenvironment and initiate survival signaling in these non-proliferating cells, establishing a state of dormancy. The protection afforded by this interaction is specific and provides a potential target for therapeutic intervention.				
14. SUBJECT TERMS Fibroblast growth factor, transforming growth factor beta, dormancy, metastasis				15. NUMBER OF PAGES 21
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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INTRODUCTION

Our work under this grant addressed two problems in breast carcinogenesis. It sought a clearer understanding of the progression from normal mammary epithelial tissue to invasive, metastatic carcinoma as it related to the expression of FGF-2 and it permitted the development of a novel paradigm for understanding the role of the bone marrow microenvironment in inhibiting the growth of and providing a survival advantage to microscopic metastases in the bone marrow. The latter data will direct the development of therapy targeting the interaction of the microenvironment with breast cancer cells at either the site of interaction or downstream signaling pathways initiated by this interaction.

As we noted in last year's annual report, the proposed staining of bone marrow slides containing micrometastases from women with breast cancer with antibodies to FGF-2, TGF beta and TGF beta receptor 2 could not be carried out. Our collaborator who had access to those slides in Germany, Stephan Braun, suddenly changed jobs and moved to Austria where he no longer had access to these samples. The work we began in the interim, however, has yielded invaluable insight into mammary carcinogenesis and the dormancy and the survival of microscopic metastases in the bone marrow.

In the first project, we continued to investigate the role of FGF-2 in the dedifferentiation of mammary ductal epithelial cells. We expanded our sample size and have stained blinded archival surgical samples from a total of 55 patients with histologies that spanned the spectrum from normal mammary ducts and lobules to invasive, metastatic breast cancer using immunofluorescence antibody labeling. These data confirm a loss of FGF-2 expression in invasive breast cancer and extend the data to show that the loss does not take place from most transformed cells while they are confined to the duct. This suggests either a potential role for regional control over FGF-2 expression that appears to diminish as cells move away from the duct or a causal effect of FGF-2 in inhibiting de-differentiation. We followed up these data with studies on the role enforced re-expression of FGF-2 in a highly de-differentiated breast cancer cell line that we previously showed to have less malignant behavior, including decreased motility and invasiveness due to overexpression of FGF-2. Our data show that expression of FGF-2 induces constitutive activation of focal adhesion complexes and rearrangement of the stress fibrils, possible mechanisms responsible for decreased motility.

The second project investigated a paradigm for inhibition of proliferation of well differentiated cells in the bone marrow microenvironment by FGF-2, upregulation of integrin expression and survival signaling initiated by ligation to fibronectin. These data instigated ongoing investigations into the survival signaling initiated by these interactions that may be potential targets of therapy.

BODY

A. Role of FGF-2 in mammary cell de-differentiation

We obtained 4 micron sections of biopsy and mastectomy specimens from the Department of Pathology and Laboratory Medicine of our institution in a blinded manner under an IRB-approved protocol that qualified for exemption because of its blinded retrospective nature. We stained all of the samples with antibodies to FGF-2 and some of the samples with antibodies to FGF receptor 1 and to cytokeratin 14 to identify myoepithelial cells. We stained specimens from 55 patients. Many slides had multiple pathologic features. Tables 1 and 2 depict our results. The reported FGF-2 staining is in epithelial cells. The data suggest that large majority of epithelial cells continue to stain for FGF-2 while in the mammary duct, regardless of their state of transformation. Once carcinomas in situ become invasive, the majority of tumors no longer stain positive for FGF-2. This suggest a possible regional influence on ductal cells even in a state of transformation by the environment of the mammary duct. Alternatively, expression of FGF-2 may

inhibit invasion of transformed epithelial cells and once lost, the cells have a higher chance for becoming invasive. All of the samples tested at all stages of dedifferentiation stained positive for FGFR1 (Table 2).

Table 1. Immunofluorescence detection of FGF-2 in mammary cells

Pathology	number of samples	number FGF-2 positive (% positive)
normal duct/lobule	43	37 (86%)
benign hyperplasia	22	22 (100%)
fibrocystic duct	20	14 (70%)
atypical hyperplasia	11	9 (82%)
carcinoma in situ	14	10 (71%)
invasive carcinoma	23	7 (30%)
fibroadenoma	6	2 (33%)
phylloides tumor	1	1 (100%)

Table 2. Immunofluorescence detection of FGF-2 and FGFR in cells of mammary duct origin

Pathology	number of samples	number FGFR positive
normal lobule	7	7
normal duct	10	10
fibrocystic duct	1	1
benign hyperplasia	2	2
atypical hyperplasia	2	2
carcinoma in situ	6	6
invasive carcinoma	14	14

The hypothesis that FGF-2 is influencing the malignant behavior of transformed mammary cells is supported by our prior publications where enforced re-expression of FGF-2 in breast cancer cells induced a more differentiated phenotype and decreased proliferation and motility (1-3). MDA-MB-231 cells enforced to re-express FGF-2 were less tumorigenic, had decreased motility and invasiveness (2) Figure 1 demonstrates the expression of FGF-2 in the transfected cells and shows an example of the inhibited motility in a patch wound assay of scatter migration (2).

Figure 1.

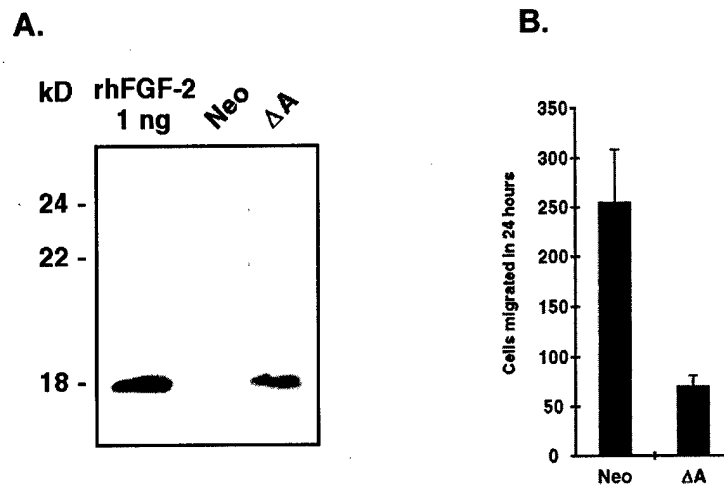


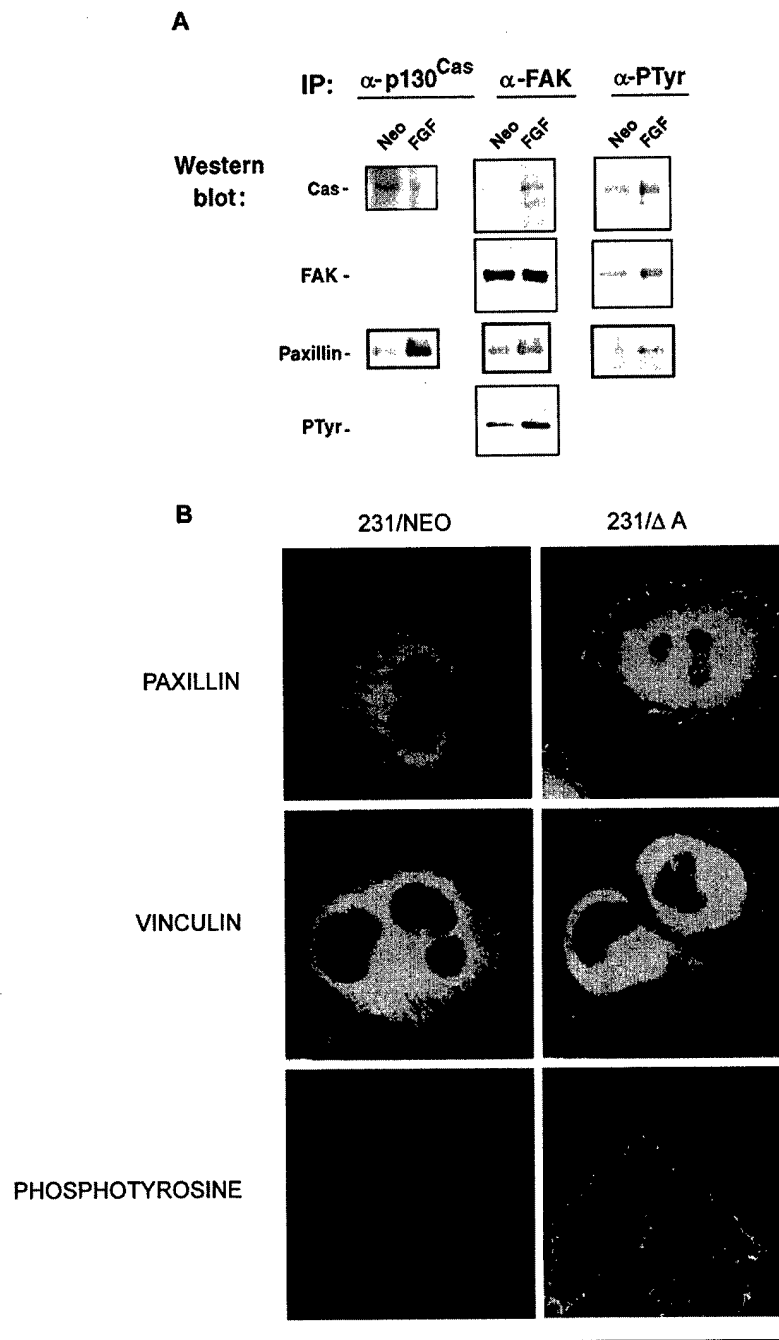
Figure 1. A. Western blot of lysates from MDA-MB-231/ΔA cells demonstrating expression of FGF-2. Neo represents MDA-MB-231 cells transfected with pCI-neo (Promega) vector that contains an SV40-promoted bacterial neomycin phosphotransferase gene (Neo) and an immediate-early CMV promoter. Cells denoted as ΔA were transfected with pCI-neo vector into which was cloned an FGF-2 cDNA cleaved with restriction endonucleases Sac II and ApaI, deleting the three CUG codons upstream of the Apa I site 11 bases upstream to the AUG start site of FGF-2, that expresses only the 18 kD isoform of FGF-2 (2). Recombinant human FGF-2 was used as a control. (B) Our prior work has demonstrated that these highly dedifferentiated breast cancer cells acquire a less malignant phenotype when enforced to express FGF-2. Among the newly acquired traits is a decreased ability to migrate (2).

FGF-2 expression promotes aberrant activation of the focal adhesion complex

We investigated potential mechanisms for decreased motility in MMDA-MB-231 cells overexpressing FGF-2. FGF-2 has been shown to modulate expression of integrins and integrins can transduce signals through clustering or ligand binding that result in the cyclic formation and dispersal of focal adhesion complexes at their cytoplasmic domains (4). To determine whether FGF-2 re-expression affects this process central to motility, we investigated the formation of focal adhesion in the cells transfected with the two vectors. Focal adhesion complex formation was determined by immunoprecipitation (IP) of complexes with antibodies to proteins localized in the complex, p130^{Cas} and FAK and to phospho-tyrosine, followed by Western immunoblotting of precipitated complexes with antibodies to p130^{Cas}, FAK and phospho-tyrosine and to paxillin, another member of the complex. Figure 2A demonstrates that complexes were constitutively assembled and members of the complex were constitutively tyrosine phosphorylated in cells expressing FGF-2. There was an increased association of p130^{Cas} with FAK, of paxillin with FAK and of paxillin with p130^{Cas} and an increase in the constitutive tyrosine phosphorylation of p130^{Cas}, FAK and paxillin, suggesting they were functionally more active in cells expressing FGF-2. Precipitation and staining with the same antibody demonstrated that the protein levels of p130^{Cas} and FAK remained unchanged with re-expression of FGF-2. These findings were verified using immunofluorescence photography that demonstrated staining for paxillin, vinculin and phosphotyrosine around the cell periphery (Figure 2B). Vinculin is a member of the focal adhesion complex that has been suggested to play a stabilizing role for members of focal adhesion complex proteins at focal contact points (5, 6). We confirmed these results using transient transfections of MDA-MB-231 cells with pCI-Neo and pCI-ΔA vectors and analysis by immunofluorescence. FGF-2-transfected cells showed a peripheral staining pattern for paxillin, vinculin and phosphotyrosine quite similar to that of permanently transfected and selected 231/ΔA

cells (Figure 2C). These data demonstrate that re-expression of FGF-2 in MDA-MB-231 cells induces constitutive stable, omnidirectional formation and activation of focal adhesion complexes.

Figure 2AB.



C.

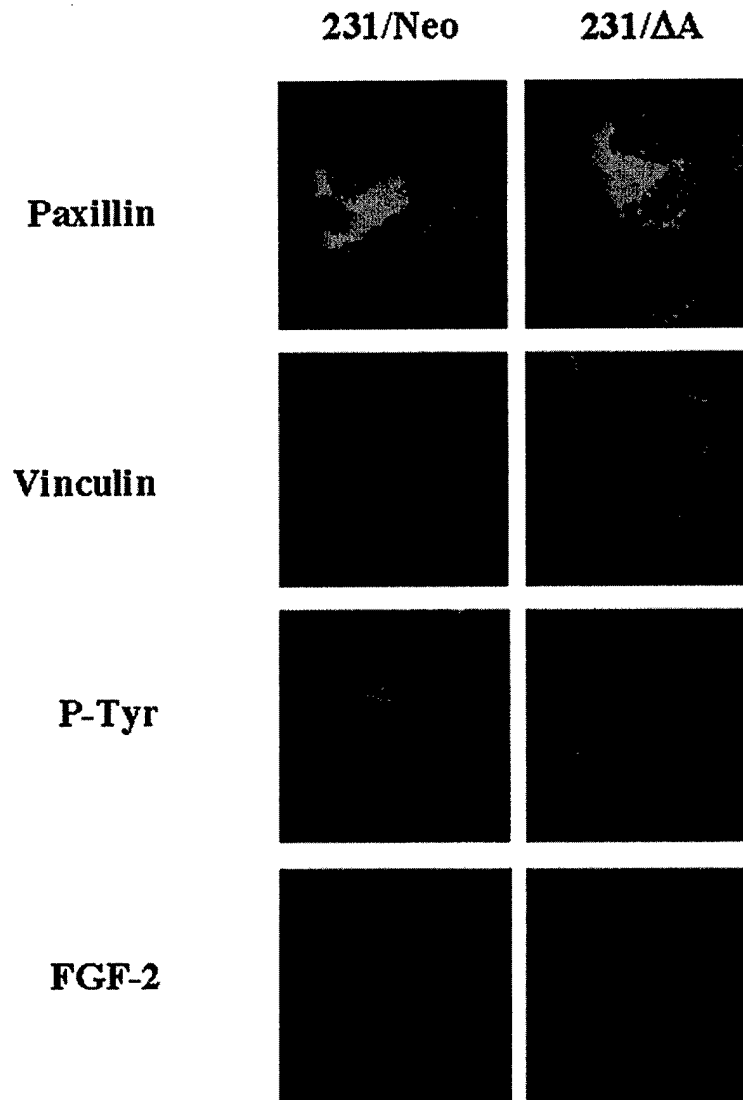


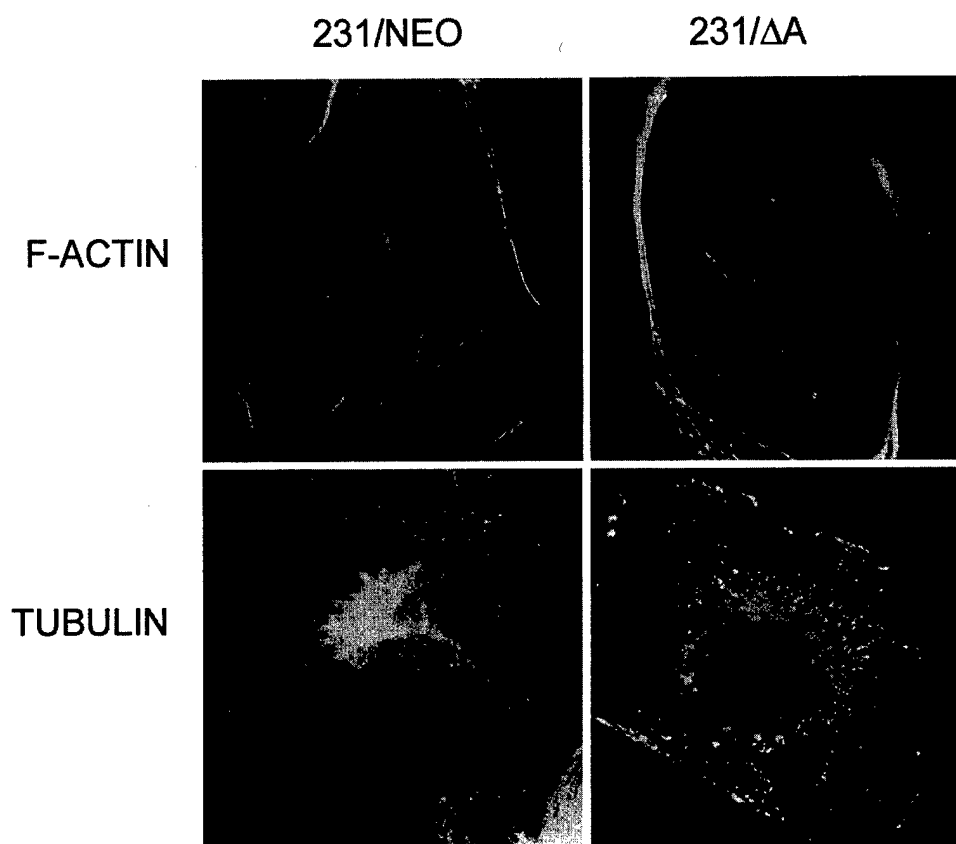
Figure 2. Expression and phosphorylation pattern of focal adhesion complex proteins p130^{Cas}, FAK and Paxillin as determined by IP-Western and immunofluorescence techniques. (A) Equal amounts of total protein from lysates of permanently transfected, selected 231/Neo and 231/ΔA cells were used for immunoprecipitation with specific primary antibodies followed by SDS-PAGE and Western blot detection. (B) Indirect immunofluorescence detection revealed increased amounts of paxillin, vinculin and phosphotyrosine in focal adhesion points near the cell periphery in 231/ΔA cells from A. (1000x magnification). C. Indirect immunofluorescence detection of FGF-2 and focal adhesion complex proteins in transiently transfected MDA-MB-231 cells. Localization of paxillin vinculin and phospho-tyrosine (p-Tyr) signals in the periphery of MDA-MB-231 cells is seen in cells transiently transfected with the pCI-ΔA vector (the pCI-Neo vector was used as a control). All cells were stained with the antibodies to antigens shown and FITC-labeled secondary antibody and with an anti-FGF-2 antibody and Texas-red stained secondary antibody. Shown is the FGF-2-stained sample additionally stained with anti-phospho-tyrosine antibody.

FGF-2-expression induces aberrant condensation of cytoskeletal proteins

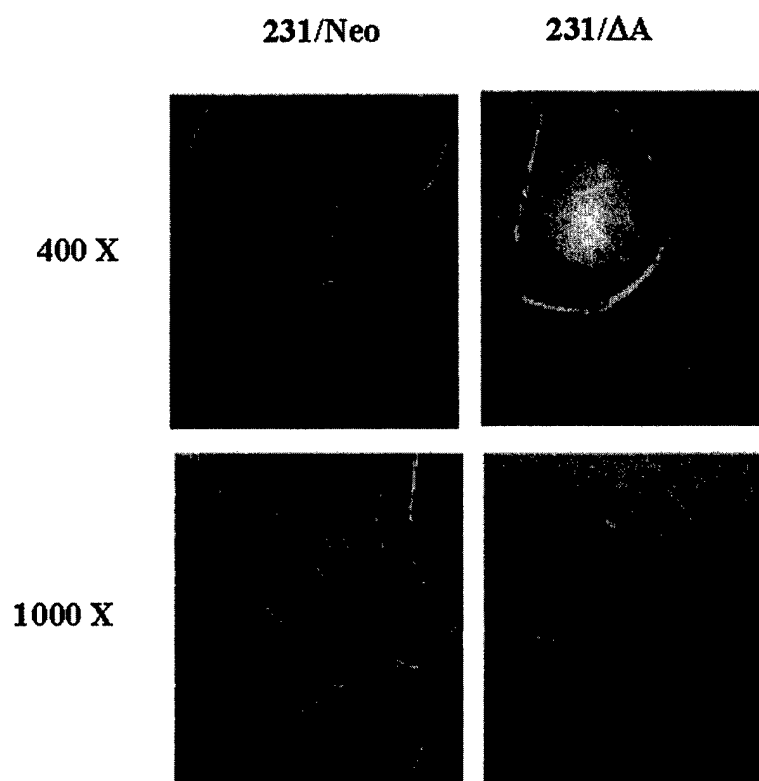
Activation of focal adhesion complexes via integrin or growth factor signaling results in dynamic changes in cytoskeletal architecture, a hallmark of cell motility (7-9). To determine if the constitutive activation of focal adhesion complexes in cells enforced to re-express FGF-2 caused changes in the cytoskeleton, we compared the organization of the cytoskeleton in 231/ Δ A and 231/Neo cells by analyzing the organization of filamentous actin (F-actin) and α -tubulin using immunofluorescence microscopy. The 231/Neo cells exhibited characteristic stress fibers that consist of bundles of F-actin (10) and a fine meshwork of α -tubulin throughout the cytoplasm, reflecting their dynamic migratory behavior (fig. 3A). The cytoskeletal architecture of FGF-2-expressing cells, however, was characterized by a drastically different organization. Stress fibers were condensed around the periphery of the cells and the α -tubulin network appeared as an array of punctate structures instead of the dynamic network pattern seen in 231/Neo cells. We confirmed the F-actin reorganization due to FGF-2 expression in MDA-MB-231 cells transiently transfected with the pCl- Δ A and control vectors. Controls exhibited a pattern of stress fiber organization very similar to that of parental MDA-MB-231 cells, while the majority of FGF-2-expressing cells demonstrated condensation of F-actin around the perimeter (fig. 3B, 400x magnification). The condensed appearance of F-actin appears to represent parallel F-actin bundles following the contour of the cell periphery (Fig 3B; 1000x magnification). The reorganization of actin stress fibers was quite dramatic both visually and quantitatively. The percentage of cells with actin stress fibers decreased from 79.5 ± 5.5 % to 10.5 ± 2.0 % ($p < 0.001$) while the percentage of cells with aberrant peripheral actin condensation increased from 26.1 ± 5.2 % to 80.5 ± 5.5 % ($p < 0.001$) in the experiment shown (fig. 3C). Similar results were obtained in repeat experiments. Since appropriate cyclic formation/dispersal of cytoskeletal structures are essential for motility, the aberrant condensation most likely contributes to the observed reduction in the migratory potential of FGF-2-expressing cells.

Figure 3.

A.



B.



C.

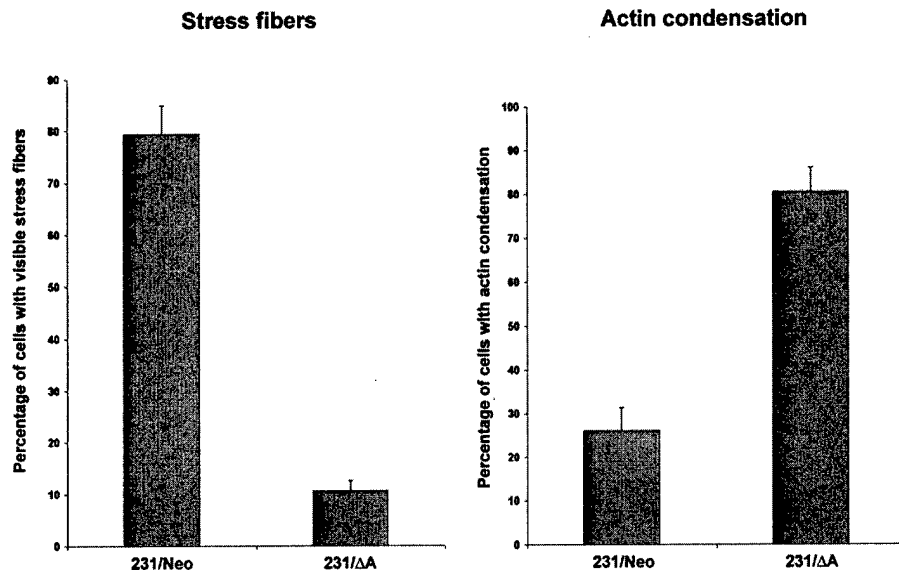


Figure 3. Immunofluorescence detection of cytoskeletal structures. A. F-actin-containing stress fibers were evenly distributed in permanently transfected 231/Neo cells while they appeared condensed near the cell periphery in 231/ΔA cells as detected by FITC-Phalloidin staining. Alpha tubulin molecules appear as punctate structures in 231/ΔA cells as detected by indirect immunofluorescence using anti-alpha tubulin primary antibody and FITC-labeled anti mouse secondary antibody (1000x magnification). B. Immunofluorescence detection of cytoskeletal structures (green) and FGF-2 (red) in MDA-MB-231 cells transiently transfected with the pCI-Neo vector (left) or the pCI-ΔA vector (right) as photographed under 400X (top) or 1000X (bottom) magnifications. C. Quantitative representation of decreases in the percentage of cells containing stress fibers and increases in cells with peripheral condensation of actin filaments in cells transiently transfected with FGF-2 and control vectors. Represented are data from one of three experiments with similar results. The data from each experiment were collected from one hundred cells positively staining for FGF-2 or one hundred Neo-transfected cells from five fields per slide. The data presented are the mean + S.D. from three slides.

In summary, our data demonstrate that intracellular expression of FGF-2 in MDA-MB-231 breast cancer cells promotes aberrant activation of focal adhesion complexes, and induces condensation of cytoskeletal structures, all of which potentially contribute to a unique differentiation-inducing role with an impaired migratory behavior.

B. A role for FGF-2 in the clonogenic potential of breast cancer cells on bone marrow stromal proteins: a paradigm for dormancy

Breast cancer cells metastasize to the bone marrow early in the course of the disease (10). Most metastatic cells die upon reaching the marrow microenvironment, but some well-differentiated cells that survive can remain dormant, or growth arrested without loss of viability, for years (11, 12). They remain protected from death and, in fact, survive multiple rounds of adjuvant chemotherapy administered specifically to eradicate them (13). While mechanisms of dormancy remain largely unknown, a variety of growth factors and ligands of cellular integrins in the marrow microenvironment may influence the fate of the metastatic cell. These factors have well-established effects on cell behavior, including protection of hematopoietic stem cells (14, 15).

Basic fibroblast growth factor (FGF-2), a growth factor endemic to the marrow microenvironment (16-20), is likely to play a significant role in initiating dormancy. It has a role in morphogenic differentiation of mammary ducts (21), inhibits growth (22-26) and induces a more differentiated state in breast cancer cells (2, 3). The antiproliferative effects of FGF-2 are restricted to well-differentiated breast cancer cells but its overall civilizing effects on malignancy extend to highly aggressive breast cancer cells (2).

Here, we present evidence that supports a paradigm in which FGF-2 initiates a more differentiated, dormant state in well-differentiated micrometastatic breast cancer cells. This encompasses cell cycle arrest and changes in the integrin repertoire. Cells with improperly ligated integrins such as $\alpha 5\beta 1$, upregulated by FGF-2 in fibroblasts and endothelial cells (27, 28), undergo cell death, likely due to ligand incompatibility (29). Ligation of integrin $\alpha 5\beta 1$ by fibronectin, a component of bone marrow stroma (30) which can initiate survival signaling (31, 32), promotes survival of FGF-2-responsive cells.

FGF-2 completely inhibited the ability of MCF-7 and T-47D cells to form growing clones, while epidermal growth factor (EGF), also abundant in bone marrow, had no such effect (Figure 4). Notably, FGF-2 did not eliminate a small number of morphologically distinct, dormant clones at the ≤ 10 cell stage. FGF-2 did not inhibit the growth of MDA-MB-231 cells, a highly de-differentiated cell line. The bone marrow stromal proteins fibronectin, collagen I and collagen IV increased the efficiency of growing clone formation in MCF-7 and T-47D cells (data not shown).

Figure 4.

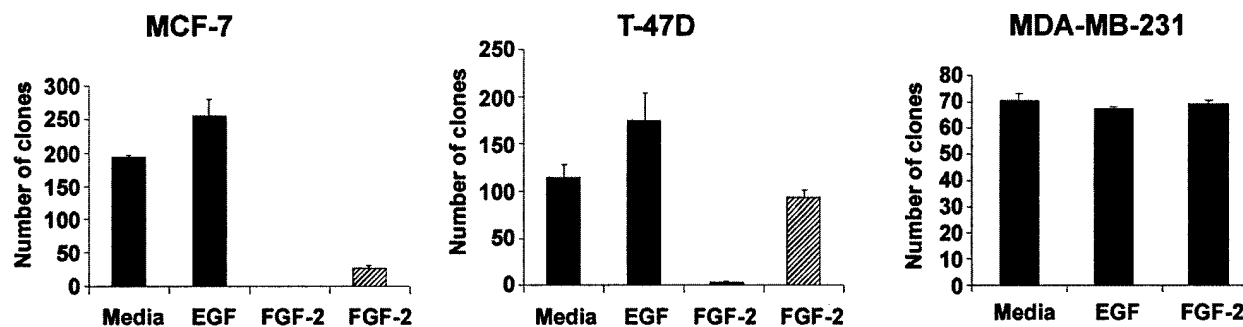


Figure 4. Effects of EGF and FGF-2 on the clonogenic potential of well and poorly-differentiated breast cancer cells in tissue culture. MCF-7 and T-47D (1,000 cells/well) and MDA MB-231 (200 cells per well) were incubated in 24 well plates \pm 10 ng/ml EFG or FGF-2 for 6 days, stained with crystal violet and clones with ≥ 29 actively growing cells (■) or with ≤ 10 well spread, growth arrested cells (▨) were counted.

Culturing the cells with FGF-2 in the presence of these stromal proteins revealed that fibronectin provided a distinct survival advantage to MCF-7 and T-47D clones of ≤ 10 cells in the presence of FGF-2 after 5 days in culture in contrast to collagen I and collagen IV (MCF-7 data shown in Figure 5A). This preferential survival was sustained for up to 15 days, with ratios of 249 and 305% for fibronectin, 55 and 57% collagen I and 32 and 99% for collagen IV for MCF-7 and T-47D cells respectively, as compared to tissue culture-treated plates (Figure 5B). The flattened morphology of dormant cells contrasted to growing cells on fibronectin underscores their altered status (Figure 5C).

Figure 5.

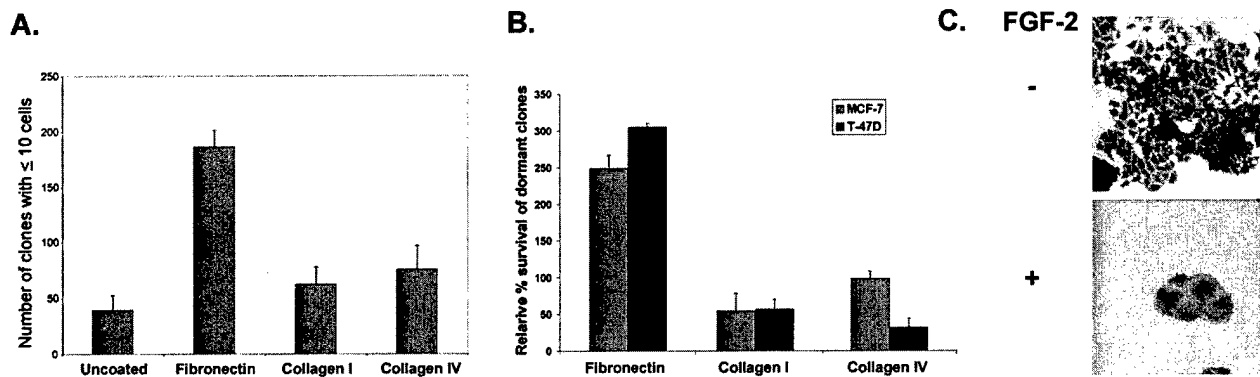


Figure 5. Fibronectin rescues the survival of FGF-2-treated dormant MCF-7 and T-47D cells. Cells were incubated in 6 well variably coated plates (5,000 cells/well) with FGF-2 10 ng/ml for 5 days (A) (MCF-7 cell data are shown) to 15 days, stained with crystal violet and colonies of ≤ 10 cells counted. B. Ratios of dormant clones on fibronectin, collagen I and collagen IV to those on tissue culture plates after 15 days. C. Differential appearance of growing and spread out dormant cells on fibronectin; 200X magnification.

To determine the mechanism, we analyzed the effects of FGF-2 on changes in the expression patterns of integrin mRNAs in MCF-7 cells cultured on fibronectin for 3 and 5 days using a gene chip microarray. Increases were observed in integrins $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 1$, $\beta 3$ and $\beta 4$ with either 3 or 5 days of incubation with FGF-2. Increases in the fibronectin receptor mRNA $\alpha 5$ and $\beta 1$ at three days and $\alpha 5$ at five days are shown in Figure 6A. There were no significant differences in $\alpha 4$, the other fibronectin receptor. FGF-2 induced marked increases in integrin $\alpha 5$ protein levels in both MCF-7 and T-47D cells (Figure 6B) that were sustained for the 5 days assayed (not shown). MDA-MB-231 cells had constitutively high $\alpha 5$ levels that did not vary with FGF-2 treatment. The increases in integrin $\alpha 5$ by FGF-2 in T-47D cells (and MCF-7 cells, not shown) were confirmed by immunofluorescence micrographs (Figure 6C). The protein levels of integrin $\beta 1$ were confirmed by Western blot in both MCF-7 and T-47D cells at 3 days (Figure 6D) and 5 days (not shown). Increased protein levels of integrins $\alpha 2$, $\beta 3$ and $\beta 4$ were also observed, while expression of integrins $\alpha 3$, $\alpha 4$, $\alpha 6$ did not vary significantly.

Figure 6.

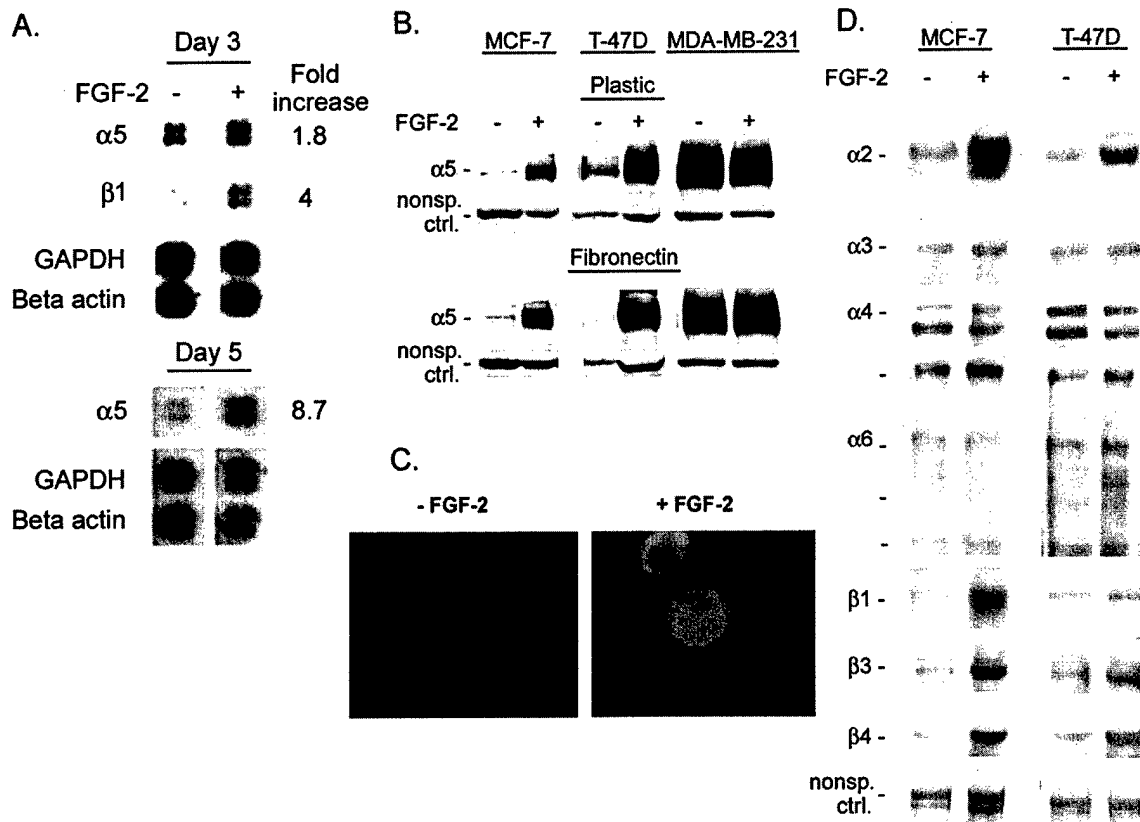


Figure 6. FGF-2 regulates expression of integrins. A. Gene chip analysis of integrin $\alpha 5$ and $\beta 1$ mRNA expression in MCF-7 cells incubated \pm FGF-2 for 3 or 5 days on fibronectin-coated plates. Densitometer quantitations normalized against GAPDH and actin mRNA standards are shown. B. Western blots of integrin $\alpha 5$ from cells incubated \pm FGF-2 for 3 days on tissue culture- or fibronectin-coated dishes. C. Indirect immunofluorescence of integrin $\alpha 5$ in T-47D cells on cover slips \pm FGF-2 10 ng/ml for 24 hours. D. Western blots of integrins $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\beta 1$, $\beta 3$ and $\beta 4$ in MCF-7 and T-47D cells incubated \pm FGF-2 for 3 days. Nonspecific bands were used as loading controls.

We sought to determine the specific contribution of integrin $\alpha 5$ to the survival of the non-proliferating clones on tissue culture-treated dishes. Function-specific blocking antibody to integrin $\alpha 5$ significantly reduced the survival of ≤ 10 cell clones in FGF-2-treated MCF-7 cells while a control blocking antibody to integrin $\alpha 3$, demonstrated no inhibition in survival (Figure 7). To demonstrate the functional specificity of fibronectin binding in rescuing survival of dormant clones, FGF-2-treated cells on fibronectin were incubated with blocking peptides to fibronectin, collagen I, laminin I and a nonspecific control. Blocking peptides P1 and P5 to fibronectin specifically reversed the fibronectin-mediated rescue of dormant clones in FGF-2 treated MCF-7 cells by 63.6% and 56.7%, respectively ($p < 0.001$ for both, Student's t test) (Figure 8A) and in T-47D cells by 50.1% and 43.4%, respectively ($p < 0.001$) (not shown). Analogous experiments on tissue culture-, collagen I- or laminin I-coated plates showed no such effects with specific blocking peptides with either cell line. Blocking the ligation of integrin $\alpha 5 \beta 1$ by P1 induced TUNEL staining, suggesting that the inhibitory effects of FGF-2 resulted in apoptosis rescuable by fibronectin ligation (Figure 8B).

Figure 7.

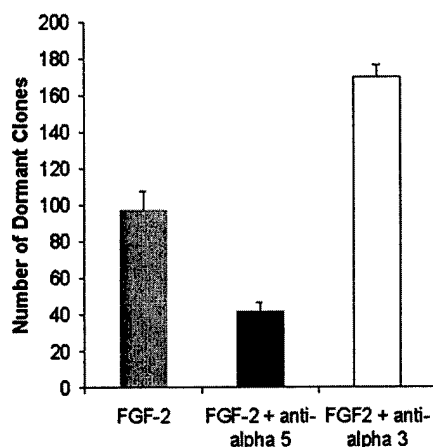


Figure 7. Integrin $\alpha 5$ supports survival of residual dormant MCF-7 clones on tissue culture-treated plates. MCF-7 cells were incubated in quadruplicate 6-well plates (5,000 cells/well) \pm 10 ng/ml FGF-2, \pm 2 μ g neutralizing antibody to integrin $\alpha 5$ or integrin $\alpha 3$, cultured for 5 days, stained with crystal violet and clones with ≤ 10 cells were counted.

Figure 8.

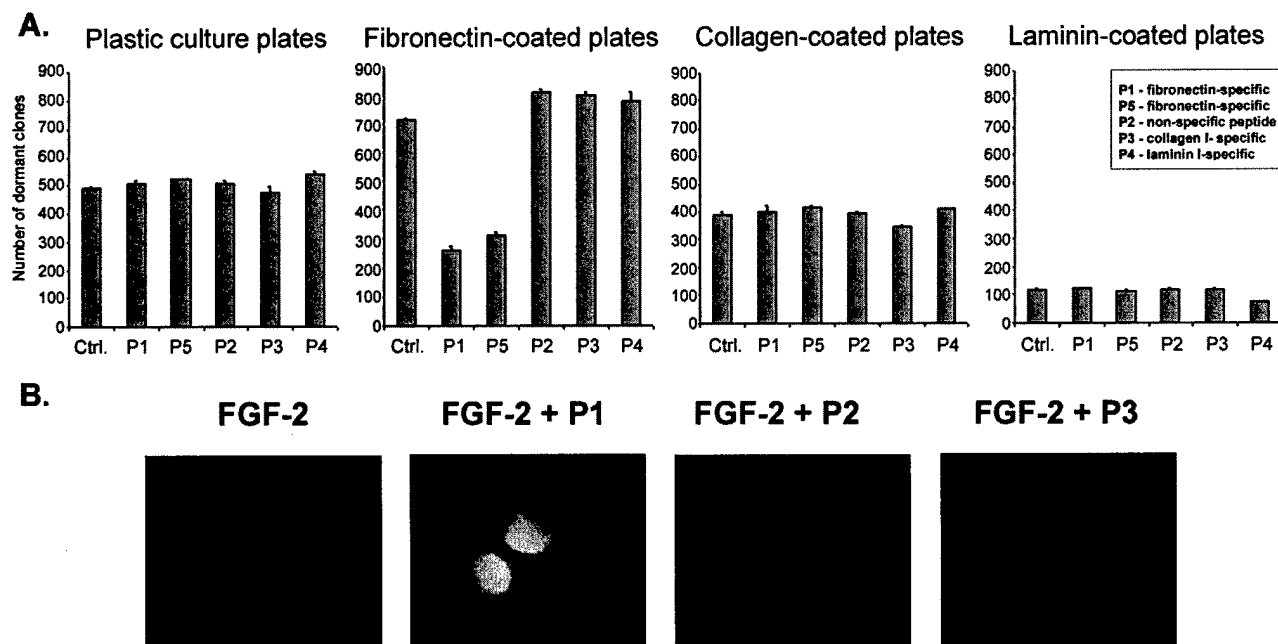
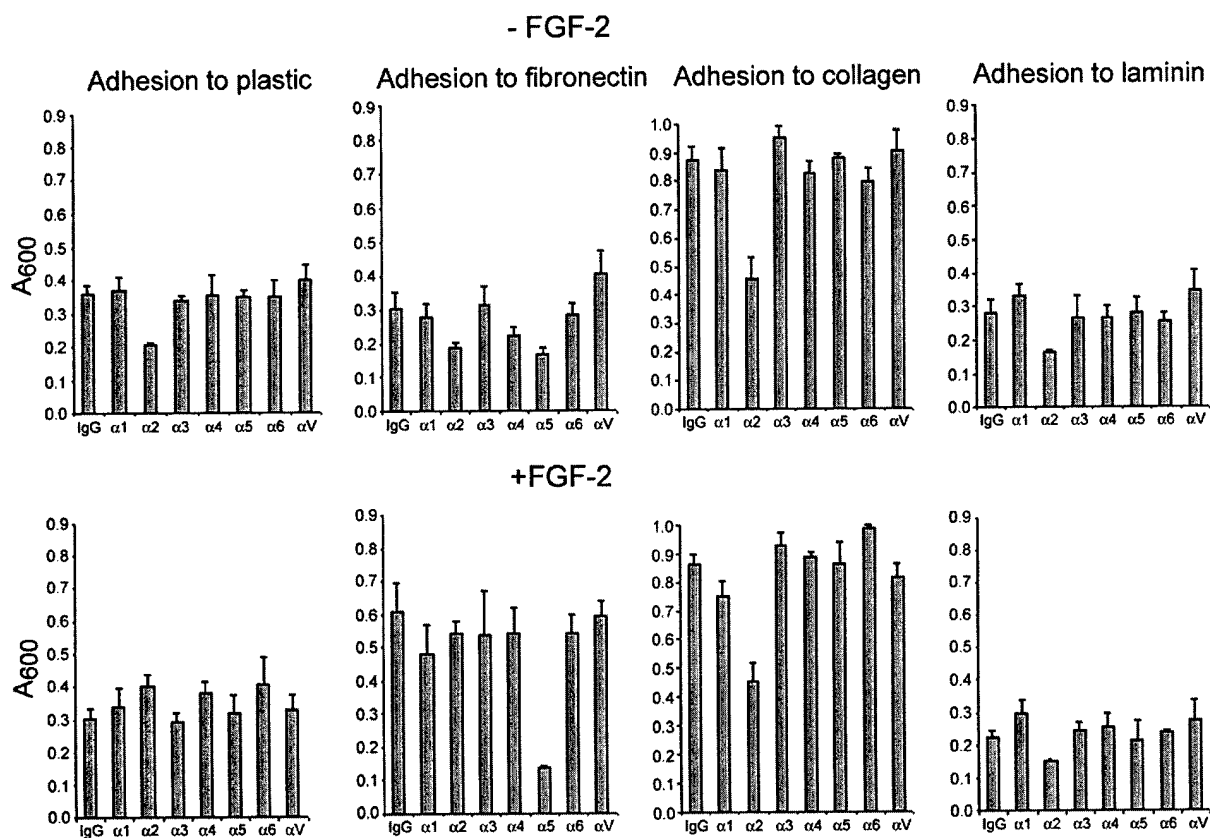


Figure 8. Ligation of integrin $\alpha 5\beta 1$ provides specific protection from cell death in well-differentiated breast cancer cells. A. MCF-7 cells (and T-47D cells, not shown) were incubated with FGF-2 on variably coated plates. Blocking peptides were added after 3 days. Colonies with ≤ 10 cells were stained with crystal violet at 6 days and counted. B. T-47D cells were incubated on fibronectin-coated plates with FGF-2 and blocking peptides were added after 3 days. Cells were probed 24 hours later with anti-integrin $\alpha 5$ antibody and Texas Red-tagged secondary antibody and assayed by TUNEL-FITC.

Control experiments demonstrated the capacity of both cell lines to adhere with specificity to all three stromal proteins. In T-47D (Figure 9A) and MCF-7 cells (not shown) inclusion of FGF-2 in 3 day cultures on plastic doubled re-adhesion to fibronectin but had no effect on adhesion to plastic, collagen or laminin. Antibody to integrin $\alpha 5$ blocked adhesion to fibronectin in FGF-2 treated cells by 75% but only inhibited untreated cell adhesion by a third. Blocking antibody to $\alpha 2$ decreased adhesion to collagen and laminin in both FGF-2-treated and untreated cells equally. While adhesion to collagen surpassed adhesion to fibronectin, it did not support dormant clone survival (Figure 8A). Similar specificity was seen in MCF-7 (Figure 9B) and T-47D cells (not shown) cultured on fibronectin and re-adhered to fibronectin or plastic. Antibody to integrin $\alpha 5\beta 1$ blocked adhesion to fibronectin in FGF-2 treated cells, while antibody to integrin $\alpha 2\beta 1$ had no effect on fibronectin adhesion. Antibody to integrin $\alpha 5\beta 1$ had no effect on adhesion to plastic, in contrast to the inhibitory effect of integrin $\alpha 5$ blocking antibody on dormant clone survival on plastic (figure 7). These data are consistent with a specific survival effect derived from ligation to fibronectin in dormant cells.

Figure 9.
A.



B.

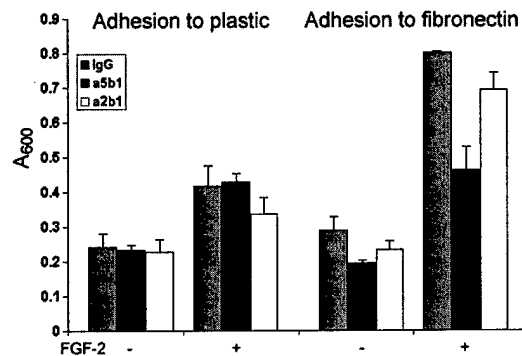


Figure 9. Adhesion of breast cancer cells to stromal proteins. Both MCF-7 and T-47D cells were cultured \pm FGF-2 on tissue culture (A) or on fibronectin-coated plates (B), detached with Cell Dissociation Solution, washed with PBS and counted. Cells were incubated with 2 μ g/ml blocking monoclonal antibodies to the integrins or mouse IgG for 30 minutes at 37°C and 50,000 cells were incubated in 24 well variably-coated tissue culture plates for 45 minutes at 37°C. Attached cells were stained with crystal violet and the A₆₀₀ of the extracted dye was measured, as described. Results were similar for both cells. Shown are data for T-47D (A) and MCF-7 cells (B).

Our data support a potential mechanism for dormancy of well-differentiated breast cancer cells metastatic to the bone marrow. In the model, cancer cells encounter FGF-2, a growth factor implicated in mammary cell differentiation, and acquire a more differentiated phenotype. New traits include growth arrest and altered expression of integrins, including upregulation of integrin $\alpha 5 \beta 1$. Most growth arrested cells die, but some survive by binding fibronectin, which likely initiates survival signaling.

Prior studies support this model. FGF-2 is abundant in the marrow (16-20) and has a spectrum of effects on mammary cells. It is implicated in mammary duct morphogenesis (21) but its expression stops with malignant transformation (33-35). Enforced re-expression of FGF-2 in breast cancer cells induces a more differentiated phenotype, including formation of branched structures in matrigel and decreased migration, invasion, growth in soft agar and tumor formation in nude mice (2, 3). FGF-2 inhibits proliferation of well-differentiated breast cancer cells in G₁ (23, 24, 36). Here, FGF-2 inhibited growing clones in well-differentiated cells but had no effect in MDA-MB-231 cells, consistent with diminished dormancy rates of poorly differentiated cells.

FGF-2 can modulate the expression of integrins, including $\alpha 5$ and $\beta 1$ (27, 28), whose ligation promotes survival in a variety of cell types (24, 25). Here, FGF-2 induced $\alpha 5 \beta 1$ expression in well-differentiated breast cancer cells, an effect not mediated by feedback upregulation through fibronectin (Figure 3B). Integrin modulation may be an element of differentiation that allows well-differentiated breast cancer cells to interact with the marrow microenvironment. Blocking appropriate ligation of integrin $\alpha 5 \beta 1$ resulted in apoptosis. This effect may be mediated through a default mechanism called integrin-mediated death (IMD) (29) and may serve to restrict the survival of well-differentiated breast cancer cells to fibronectin-rich compartments of the marrow.

These experiments suggest a potential mechanism for dormancy and resistance to death in breast cancer cells in the bone marrow and present the integrin $\alpha 5 \beta 1$ -fibronectin interaction as a potential target for disrupting this resistance. Studies addressing survival signaling will reveal additional targets for therapy.

KEY RESEARCH ACCOMPLISHMENTS

1. FGF is expressed in a vast majority of cases in normal ductal and lobular epithelial cells, benign hyperplasia, fibrocystic disease, atypical hyperplasia and carcinoma in situ but its expression markedly drops in frequency in invasive cancer.
2. FGF receptor 1 is expressed in all stages of mammary duct dedifferentiation to cancer – preliminary data.
3. Enforced re-expression of FGF-2 in poorly differentiated breast cancer cells results in a less malignant phenotype, including decreased motility. The decreased motility is associated with constitutive omnidirectional focal adhesion activation and rearrangement of the cytoskeleton.
4. FGF-2 inhibits survival and clonogenic potential of well-differentiated breast cancer cells in vitro inducing a dormant phenotype after undergoing two or three cell divisions.
5. FGF-2 upregulates a number of integrins including integrins $\alpha 5$ and $\beta 1$ in well-differentiated breast cancer cell lines.
6. Ligation of integrin $\alpha 5 \beta 1$ by fibronectin specifically imparts survival signaling and partially restores clonogenicity of FGF-2-treated cells.

REPORTABLE OUTCOMES

Wieder R, Boots M, and Korah R. FGF-2 induces breast cancer cell survival on fibronectin: a paradigm for breast cancer dormancy. The Department of Defense Breast Cancer Research Program Meeting, "Era of Hope". Orlando, FL, September 2002, # 25-23.

R. Korah, M. Boots, Q. Wang, R. Wieder. Flavopiridol inhibits Akt-signaling and blocks survival of dormant breast cancer cells. Proceedings of the American Association for Cancer Research 2003, v. 44, 2nd ed., p. 409, #2065.

91. M. Boots, and R. Korah A model for metastatic breast cancer dormancy through fibronectin-mediated survival signaling. Program Proceedings of American Society of Clinical Oncology, 2003, v. 22. p. 23, # 89. (Selected for poster discussion).

92. Robert Wieder, Monika Boots, and Reju Korah. Integrin $\alpha 5 \beta 1$ promotes survival of growth-arrested breast cancer cells: an *in vitro* paradigm for breast cancer dormancy in bone marrow. (2003) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, p. 33, #E2 (Selected for oral presentation}.

CONCLUSIONS

1. FGF-2 is associated with morphogenic differentiation in mammary duct differentiation in puberty and late pregnancy. Its expression is lost in malignant transformation. Our data suggest that the loss of expression does not occur until cancer cells have become invasive. To differentiate between cause or effect, enforced re-expression of FGF-2 in poorly differentiated (and well differentiated) breast cancer cell lines induced a less malignant phenotype. This was associated with decreased motility. Our studies have shown that re-expression of FGF-2 induces constitutive focal adhesion activation in these cells and aberrant rearrangement of actin filaments, likely contributing to the decreased motility.

2. We developed an in vitro model of dormancy in microscopic breast cancer metastases in the bone marrow whereby FGF-2 inhibits proliferation and hinders survival of these individual cells. It

causes an increased expression of integrins $\alpha 5 \beta 1$ which in turn, when bound to fibronectin, also abundant in the bone marrow microenvironment, promote survival of non-proliferating cells, thus establishing a dormant state. The survival protection afforded by this interaction is specific and is not merely the effect of adhesion, since adhesion to other substrata does not recapitulate the effect. Specific signaling from this interaction is being investigated as potential targets for future therapy.

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APPENDICES

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